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(54) Title: MEIOSIS ACTIVATING STEROL AUGMENTS IMPLANTATION RATE

(57) Abstract

The present invention relates to the use of a new principle for improving the viability and pregnancy potential of oocytes and pre–embryos obtained in connection with *in vitro* fertilisation and pre–embryo transfer treatment. More specifically, improvement by raising the content of Meiosis Activating Sterols (MAS) in the medium where the *in vitro* fertilisation takes place. This is achieved by exposing and culturing one or more oocytes with spermatozoa in a culture medium comprising at least one meiosis activating sterol (MAS), a MAS analogue, and/or an additive or additives capable of endogenous stimulation of the accumulation of at least one MAS. Preferred additives are FSH and EGF.

MEIOSIS ACTIVATING STEROL AUGMENTS IMPLANTATION RATE

Field of invention

The present invention relates to the use of a new principle for improving the viability and pregnancy potential of oocytes and pre-embryos obtained in connection with *in vitro*

5   fertilisation and pre-embryo transfer treatment. More specifically, the application relates to improvement of the pregnancy potential of oocytes and pre-embryos by raising the content of Meiosis Activating Sterols (MAS) in the medium where at least the *in vitro* fertilisation takes place and preferably also in the medium wherein the oocytes are cultured prior to *in vitro* fertilisation.

10

Background of the invention

Couples seeking infertility treatment, will, almost without exception, be subject to fertilisation *in vitro*, wherein the meeting between an oocyte and a spermatozoa takes place *in vitro*. The woman is most commonly receiving treatment with exogenous

15   hormones in order to regulate and stimulate the ovaries to develop more than the usual one preovulatory follicle which is seen during the natural menstrual cycle. Part of the treatment involves retrieving the oocytes from the preouvlatory follicles of the ovaries in order for the oocytes to be matured and/or fertilised *in vitro*. After fertilisation and pre-embryo development, one to three pre-embryos are replaced to the woman's uterus, and

20   she thus has the possibility of becoming pregnant and carrying her own child. This is now an established treatment, which has been performed on a large scale for more than 20 years.

Although the likelihood of achieving a pregnancy has increased over the years, the

25   frequency with which the retrieved oocytes undergo fertilisation and pre-embryo development has remained remarkably constant around 60-70%. However, only around 15 to 25 percent of the replaced pre-embryos implant and develop into offspring. Therefore, only a fraction of the retrieved oocytes will result in a conceptus. The reason why only such a small fraction of the *in vitro* developing pre-embryos possesses the

30   capacity to implant is largely unknown, but several explanations have been suggested (Salha et al., 1998): the follicles/oocytes lack sufficient maturation; the follicle/oocyte has been exposed to sub-optimal concentrations of gonadotropins; the culture conditions of

the oocyte may not reflect the conditions of the luminal fluid within the female reproductive tract; the oocyte may suffer from sub-optimal culture conditions.

5   In its natural surroundings, the oocyte is expelled from the follicle in a sea of follicular fluid which surrounds the oocyte during the first time in the fallopian tube. The follicular fluid is mixed with secretions from the fallopian tube, defining the environment in which fertilisation takes place. The follicular fluid contains a number of substances which are believed to enhance oocyte nuclear and oocyte cytoplasmatic maturation. Recent reports show that particularly follicle-stimulating hormone (FSH) and epidermal growth factor

10 (EGF) participate in these maturation processes. They are both present in follicular fluid and the fallopian tube synthesise EGF particularly around ovulation (Morishige et al. , 1993).

Mammalian oocytes are arrested in the prophase of the first meiotic division characterised

15 by the presence of the nuclear membrane, and/or germinal vesicle (GV). When the oocyte resume meiosis it is visualised by the germinal vesicle break down (GVBD), also termed oocyte maturation. In the follicle, the oocytes stay in the GV stage as the result of the maturation inhibiting effect of hypoxanthine (HX) and other purines present in follicular fluid. Oocytes in culture also remain in the GV stage when cultured in the presence of

20 physiological concentrations of HX and resume meiosis if HX is removed. The inhibitory effect of HX on the resumption of meiosis in cultured mouse cumulus enclosed oocytes, as well as cumulus deprived, naked oocytes, can be overcome by adding meiosis activating sterols, e.g. FF-MAS (4,4-dimethyl-5α-cholesta-8,14,24-triene-3β-ol) or T-MAS (4,4-dimethyl-5α-cholest-8,24-diene-3β-ol). FF-MAS was isolated and characterised from

25 human follicular fluid and T-MAS from bull testis (Byskov et al., 1995). The sterols are intermediates in the cholesterol biosynthetic pathway and are immediate products of lanosterol (Schroepfer et al., 1972) (Fig. 1).

The synthesis of FF-MAS from lanosterol is catalysed by cytochrome P450 lanosterol 14α

30 -demethylase (P45014DM) encoded by the CYP51 gene. FF-MAS is converted to T-MAS by the activity of sterol 14-reductase (Δ14R). The drug AY9944-A-7 (AY), which in the 1950s was used to lower plasma cholesterol, selectively inhibits the activity of Δ14R. The chemical structure of AY is totally unrelated to sterols and the intermediates in the cholesterol biosynthesis (for review: Mercer, 1993). Several studies have shown that

35 cholesterol producing cells accumulate 4,4-dimethyl-5α-cholesta-8,14,24-triene-3β-ol (i.e.

FF-MAS) when cultured in the presence of AY. It was shown that AY (0.1μM) acts as a competitive inhibitor of the Δ14R in cultured rat hepatocytes.

Physiological concentrations of FSH have been shown to promote oocyte maturation in
5   mice *in vitro*, and also in oocytes in germinal vesicle stage of human and monkey oocytes (Schramm and Bavister 1995). It has been shown that FSH stimulates cumulus cells to synthesise substances which positively promote maturation (Byskov et al. , 1997). However, a recent study indicated that FSH reduced the rate of triploidy in a group of pre-embryos grown in the presence of FSH, suggesting a specific role of FSH in the final
10   stages of meiosis (Merriman et al. , 1998). Furthermore, by replacing these pre-embryos to pseudopregnant mice it was shown that the number of implantations were enhanced in the group with FSH present in the oocyte maturation medium compared to the control medium and almost similar to that of *in vivo* matured oocytes.

15   EGF has been shown to enhance oocyte maturation in a number of species like the cow, pig, mouse and rat, and one study found that FSH seems to up-regulate the expression of EGF receptors in rat granulosa cells (Maruo et al. 1993). EGF promotes the ability of mouse two-cells to develop into blastocysts (Morishige et al. , 1993). In human oocytes results are less clear. A 6 h exposure of EGF in concentrations of 1 and 10 ng/ml was not
20   reflected in augmented fertilisation rates of oocytes obtained from an IVF program (Gòmez et al. 1993), whereas Goud et al. (1998) found that EGF (2 ng/ml) enhanced the number of germinal vesicle oocytes reaching metaphase II (MII) after a 30 h culture and concluded that EGF improved the nuclear and cytoplasmatic maturation of human oocytes *in vitro*.
25
During the established treatment, the *in vitro* fertilisation takes place in a basal medium (Salha et al., 1998; Trounson and Gardner 1993). In such basal medium, the oocytes are devoid the natural environment of the follicular fluid. Thus, e.g. Merriman et al. 1998 wash the oocytes, cultured in a medium with FSH and EGF prior to transfer to a basal medium
30   wherein *in vitro* fertilisation takes place.

Detailed disclosure of the invention

One problem solved by the present invention is the very low rate of implantation of the in vitro created pre-embryos. This is achieved by exposing and culturing one or more
35   oocytes with spermatozoa in a culture medium, the culture medium comprising at least

one meiosis activating sterol (MAS), a MAS being any sterol in the metabolic pathway between lanosterol and cholesterol, a MAS analogue, and/or an additive or additives capable of endogenous stimulation of the accumulation of at least one MAS.

5 The culture medium wherein the actual *in vitro* fertilisation takes place subject to the present invention is based on the finding presented in the examples. Thus, in example 2 it is demonstrated that the implantation rate in humans is significantly higher in the group of oocytes/pre-embryos exposed to a medium containing FSH and EGF during *in vitro* fertilisation compared to those exposed to the control medium. Furthermore, the presence

10 of FSH and EGF causes an accumulation of FF-MAS and T-MAS in the culture medium in which oocytes have grown as compared to the control medium. In example 1 it is demonstrated that the presence of FSH and EGF in cultures of cumulus enclosed oocytes in mice causes the accumulation of endogenous produced FF-MAS and T-MAS by attenuating the conversion of T-MAS to cholesterol.

15
Examples 1 and 2 not only show that the implantation rate is markedly increased when the *in vitro* fertilisation is performed in a medium with EGF and FSH in concentrations around those observed *in vivo*, but also substantiate that the underlying mechanism is the endogenous production of MAS and the other examples underline these findings.

20 Example 3 demonstrates that FSH and EGF enhance oocyte maturation of mouse oocytes *in vitro* and that a combination of FSH and EGF seems to enhance the effect. Example 5 shows that an inhibitor of sterol 14-reductase (AY9944-A-7) stimulates oocyte maturation of cumulus enclosed mouse oocytes in a dose-dependent way, and that the presence of the AY compound in cultures of cumulus enclosed mice oocytes causes the

25 accumulation of endogenous produced FF-MAS and T-MAS by attenuating the conversion of T-MAS to cholesterol. These findings demonstrate for the first time that cumulus oocyte complexes of mice are able to produce and accumulate FF-MAS in the presence of AY. Similar results are obtained with a cholesterol biosynthesis inhibitor, Amphotericin B, in example 6.

30
Therefore, enhancing the endogenous production of MAS represents a new mechanism to augment the pregnancy potential of pre-embryos fertilised *in vitro*.

Thus, one aspect of the invention relates to a method for *in vitro* fertilisation comprising

35 the step of exposing and culturing one or more MII oocytes with spermatozoa in a culture

medium, the culture medium comprising at least one meiosis activating sterol (MAS), a MAS being any sterol in the metabolic pathway between lanosterol and cholesterol, a MAS analogue, and/or an additive or additives capable of endogenous stimulation of the accumulation of at least one MAS. It is preferred that the *in vitro* fertilisation is *in vitro*

5   fertilisation of human oocytes.

The *in vitro* fertilisation procedure is preferably performed as follows: the intact cumulus-oocyte complexes are localised in the follicular aspirate and transferred to the culture medium comprising at least MAS, a MAS analogue, and/or an additive or additives

10   capable of endogenous stimulation of the accumulation of at least one MAS. After a few hours, purified spermatozoa are added to the cumulus-enclosed-oocyte and *in vitro* fertilisation takes place, which the next day (after 16-24 hour of culture) can be visualised by the presence of two pronuclei in the oocyte. The oocyte with two pronuclei is termed zygote. A continued culture for another 24-48 hours usually results in the first few mitotic

15   divisions of the zygote, in which case the term pre-embryos is now used. Thus, a pre-embryo consists of at least 2 cells (those two cells are called blastomers). Quite often 48-72 hours after the oocyte has been retrieved, the presence of pre-embryos is observed after the *in vitro* fertilisation and is followed by replacement of the pre-embryos to the woman's uterus for further development.

20

In one embodiment of the invention the exposure and culture of the oocytes last until zygotes and/or pre-embryos are formed. The formation of zygotes and/or pre-embryos usually occur within 16-24 hours. In a related embodiment the one or more oocytes exposed and cultured are oocytes in Metafase II (MII).

25

By MII is understood an oocyte which has gone through germinal vesicle break down, with the first polar body present and more or less expanded cumulus complex. These oocytes are readily recognised by the person skilled in the art of handling oocytes. Oocytes in MII are the type of oocytes prepared and ready to be fertilised by the sperm cell.

30

One embodiment of the present invention relates to a method for *in vitro* fertilisation comprising the steps of:
   (a) culturing one or more GV oocytes in a culture medium, the culture medium comprising at least one meiosis activating sterol MAS, a MAS analogue, and/or an additive or

35   additives capable of endogenous stimulation of the accumulation of at least one MAS;

hereby forming one or more MII oocytes;

(b) exposing and culturing the one or more MII oocytes of step (a) with spermatozoa in a culture medium, the culture medium comprising at least one MAS, a MAS analogue, and/or an additive or additives capable of endogenous stimulation of the accumulation

5   of at least one MAS;

the exposure and culturing lasting at least until zygotes and/or pre-embryos are formed.

In one aspect of the invention this method is carried out with the same medium in step (a) and step (b). Hereby the natural surroundings are maintained throughout the process and

10 a washing step is omitted. In another aspect of the invention a washing step is introduced between step (a) and step (b).

The additive or additives capable of endogenous stimulation of the accumulation of at least one MAS (e.g. FSH and EGF) may affect maturation positively and provide the

15 cumulus-oocyte complex with a meiosis promoting effect by two different mechanisms. One may be through a positive stimulus on the cumulus cells which affect the oocyte directly. The cumulus cells may also release substances to the medium, which are able to induce resumption of meiosis and sustain further maturation of other oocytes in the medium. It is therefore yet another aspect of the present invention to improve implantation

20 rate, the fertilisation rate, and/or the viability of the oocytes by the co-culture of several cumulus-oocyte complexes together in the same culture medium with the additive or additives. Those oocyte-cumulus complexes which, during the course of ovarian stimulation, have experienced a sub-optimal maturation or may be unable to resume meiosis in a physiological correct way, can in vitro receive a positive stimulation by the

25 release of substances from the more mature oocyte-cumulus complexes after the stimulation by the co-culture of several oocyte-cumulus complexes together in the presence of the additive or additives. An increase in viability of co-culture and addition of the additive or additives to the culture medium will result in a more optimal maturation of those oocytes, which during the course of ovarian stimulation lack a proper stimulus to

30 resume meiosis. As the MAS endogenously produced by the optimal matured cumulus enclosed oocytes is released to the medium, the less optimal matured oocytes will benefit from this stimulus and be induced to resume meiosis. The fertilising capacity of the retrieved oocytes will increase, and it is possible to increase the rate of fertilisation. When more viable pre-embryos are created by in vitro fertilisation in the medium with the

additive or additives the likelihood of conceiving will increase in connection with infertility treatment.

The raise in content of MAS in said media with 1) naturally occurring hormones such as a
5   glycoprotein with follicle stimulating activity (i.e. follicle stimulation hormone (FSH)), a mixture of specific FSH isoforms with an isoelectric point above 5.0, and growth factors with activity like epidermal growth factor (EGF), or with 2) substances which interfere in the biosyntetic pathway, which converts lanosterol to cholesterol in such a way that intermediates accumulate leading to an augmented MAS concentration. One aspect of the
10   invention relates to the effect of the above mentioned substances, which increase the ovarian follicular cumulus cell production of MAS and thereby enhance the capacity of the oocyte to undergo normal pre-embryo development resulting in augmented implantation and conceptional potential of the derived pre-embryos obtained during a culture period as used in connection with assisted reproduction and infertility treatment.
15

It is presently contemplated that the endogenous production of MAS takes place in the cumulus cells connected to and surrounding the oocytes. Thus, in one embodiment of the invention oocytes with no or few cumulus cells are exposed and cultured with
20   spermatozoa in a culture medium comprising MAS or a MAS analogue the implantation rate for the pre-embryo resulting from the *in vitro* fertilisation is increased. In another embodiment, cumulus enclosed oocytes are exposed and cultured with spermatozoa in a culture medium comprising MAS or a MAS analogue the implantation rate for the pre-embryos resulting from the *in vitro* fertilisation is increased.
25

In a preferred embodiment of the invention the culture medium comprises an additive or additives capable of endogenous stimulation of the accumulation of at least one MAS. In a related aspect of the invention, 2-30 oocytes are cultured and exposed together, such as 2-25, 2-20, or just 2-15 oocytes, wherein a few of these (such as less than 50%, e.g. less
30   than 40%, less than 30%, or less than 20% of the oocytes) are cumulus enclosed.

Another major aspect of the invention relates to the use of a culture medium comprising at least one meiosis activating sterol (MAS), a MAS being any sterol in the metabolic pathway between lanosterol and cholesterol, a MAS analogue, and/or an additive or
35   additives capable of endogenous stimulation of the accumulation of at least one MAS for *in vitro* fertilisation, the pre-embryo resulting from the *in vitro* fertilisation having an improved implantation rate *in vivo*.

Yet another embodiment relates to the use of at least one meiosis activating sterol (MAS), a MAS being any sterol in the metabolic pathway between lanosterol and cholesterol, a MAS analogue, and/or an additive or additives capable of endogenous stimulation of the

5 accumulation of at least one MAS in cumulus enclosed oocytes for the preparation of a culture medium for *in vitro* fertilisation, the pre-embryo resulting from the *in vitro* fertilisation having an improved implantation rate *in vivo*.

The term meiosis activating sterol (MAS) refers to a substance which is an intermediate in

10 the cholesterol biosynthesis between lanosterol and cholesterol (see fig. 1). Two examples of MAS are FF-MAS (4,4-dimethyl-5α-cholesta-8,14,24-triene-3β-ol) and T-MAS (4,4-dimethyl-5α-cholest-8,24-diene-3β-ol). A MAS will preferably be a sterol. Examples of MAS compounds are mentioned in WO96/00235, WO96/27658, WO97/00884, WO98/28323, WO98/54965 and WO98/55498. It is preferred that the MAS

15 is selected from the group consisting of FF-MAS, T-MAS, 1-methyl-zymosterol, and zymosterol.

In another embodiment a MAS analogue is added to the medium. A MAS analogue is defined as a substance causing an effect comparable to the published MAS effects. This

20 could be the published MAS effect on oocyte maturation (Hegele-Hartung et al., 1999) or GVBD (Byskov et al., 1995) or, preferably, on implantation rate as described in example 2. More specifically, MAS and MAS analogues are compounds which in the test described in Example 7 below has a percentage germinal vesicle breakdown (hereinafter designated GVBD) which is significantly higher than the control. Preferred MAS and MAS analogues

25 are those having a percentage GVBD of at least 50%, preferably at least 80%.

One aspect of the invention relates to the effect of the additive or additives which increase the ovarian follicular cumulus cell production of MAS and thereby enhance the capacity of the oocyte to undergo normal pre-embryo development resulting in augmented

30 implantation and conceptional potential of the derived pre-embryos obtained during a culture period as used in connection with assisted reproduction and infertility treatment. The additive or additives are defined as the additive or additives which lead to a ratio of at least 2 between the relative content of MAS in cumulus enclosed oocytes cultured in the presence of the additive or additives, the relative content of MAS in

35 cultured cumulus enclosed oocytes being determined by stimulating female mice with

exogenous gonadotropins 48h prior to removal of the ovaries from the mice and
recovering cumulus enclosed oocytes from the ovaries by puncturing individual follicles
and culturing the recovered cumulus enclosed oocytes in an α-MEM medium
supplemented with 3mg/l bovine serum albumin, 5 mg/l human serum albumin, 2mM L-

5   glutamin, 100IU/ml penicillin, 100μg/ml steptomycin, 4mM hypoxanthine and ^{3}H-
mevalonat for 24h at 37°C, 100% humidity and 5% CO_2 in air, followed by acidification
with 50μl 0.3M Na_2PO_4 pH=1, organic extraction three times with a five-fold surplus of n-
heptane:isopropanol (3:1 v/v), purification of MAS from the organic phase by HPLC and
determination of the ratio of radioactivity per cumulus enclosed oocyte between cumulus

10 enclosed oocytes cultured in the presence of the additive or additives and cumulus
enclosed oocytes cultured without the presence of the additive or additives.

It is preferred that the additive is selected from the group consisting of gonadotropins such
as FSH and analogues, growth hormones such as EGF and analogues, cholesterol

15 synthesis inhibitors such as compounds inhibiting sterol Δ14-reductase e.g. AY9944-A-7,
or compounds inhibiting 4-demethylase converting T-MAS to Zymosterol, compounds
activating cytochrome P450 lanosterol 14α -demethylase, and compounds with an
amphotericin like effect.

20 The term FSH refers to proteins with follicle-stimulating activity comprising the amino acid
sequences of the heterodimers of the FSH and the chains of pituitary derived proteins.
However, the term FSH is also intended to refer to substances activating the FSH-
receptor of the ovarian cumulus cells. Such substances are able to activate the FSH
receptor located on the cumulus cells, whereby resumption of meiosis is initiated.

25 Examples of such substances may be oligo peptides, derived from the entire sequence of
the FSH molecule, or derivatives thereof.

The term EGF refers to all proteins which activate the EGF receptor (e.g. EGF and
transforming-growth factor-α) and causes accumulation of MAS in the cumulus-oocyte

30 complex. Other substances which are likely to posses similar activities include substances
like activin, insulin-like growth factor I and insulin-like growth factor II.

The term cholesterol synthesis inhibitors refers to substances which interfere with the
cholesterol biosynthesis in a way which results in accumulation of meiosis activating

35 sterols. AY 9944 and Amphotericin are examples of such compounds.

It is preferred that the additive is a combination of a gonadotropin and a growth hormone, such as a combination of EGF and FSH. However, data suggest that EGF and FSH individually are capable of endogenous stimulation of the accumulation of at least one

5 MAS. It is preferred that the concentration of EGF is between 1 and 10ng EGF/ml, such as 9 ng/ml, 8 ng/ml, 7 ng/ml, 6 ng/ml, 5 ng/ml, 4 ng/ml, or 3 ng/ml, preferably 2 ng EGF/ml.

It is a further aspect of the present invention that the combination of EGF and FSH has a
10 synergistic effect on the implantation rate and on oocyte maturation.

As illustrated in example 4, less and mid-acidic FSH isoforms with a pI above 5.0 are significantly more potent in inducing mouse cumulus enclosed oocytes to resume meiosis in vitro than acidic FSH isoforms. It is therefore preferred that one additive is FSH,
15 wherein FSH is an FSH isoform with an isoelectric point above 5.0. The term FSH isoforms refers to proteins with an amino acid sequence of FSH, but which differ in their oligosaccharide structures, including the degree of terminal sialylation and/or sulfation, resulting in different isoelectric points pI's (i.e. the pH value where the net charge of the protein is zero). FSH isoforms are obtained by enzymatic or chemical modification or,
20 preferably by chromatografocusing of naturally occurring unfractionated FSH. Carbohydrate chains are removed with such treatments without affecting the amino acid sequence. Examples of such treatments are Hydrogen Fluoride and enzymatic neuraminidase treatment resulting in partial desialylation.

25 FSH is derived from naturally occurring FSH such as FSH extracted from urine, or from recombinant FSH. The preferred concentration of FSH is between 2 and 200IU FSH/l, such as between 5 and 50IU FSH/l, e.g. 50 IU/l, 40 IU/l, 30 IU/l, 20 IU/l, or 10 IU/l, preferably 25 IU FSH/l.

30 One aspect of the present invention relates to a combination of additives such as FSH and EGF with a MAS or a MAS analogue. This embodiment is especially preferred when the in vitro fertilisation is performed in a medium covered with oil. Typically the medium is water soluble and the oil, e.g. mineral oil is water insoluble.

aqueous

It is preferred that a simple medium consisting mainly of various salts is used as the starting point. Examples of simple media are M16, EBSS, Ham-F10, Whitten, Brinster, BWW, T6, Earle's, HTF, CZB, MTF, P1 and Menezo's B3 medium. It is well known for the person skilled in the art how media are prepared for a detailed description reference is

5 made to Trounson and Gardner, pages 98-101.

It is also preferred that the medium comprises antibiotics (such as penicillin and/or steptomycin), and Human Serum Albumin (HSA). It is also preferred that the culture medium further comprises a pH regulatory component to maintain the pH between 7.3

10 and 7.5. A preferred example of a pH regulatory component is bicarbonate. It is even further preferred that the culture medium has an osmolarity of 280-300 mOsmol/kg. As will be readily acknowledged by the person skilled in the art, it is preferred that the medium also contains pyruvate.

15 The term culture medium reproduction refers to a medium in which *in vitro* fertilisation and pre-embryo culture can be performed. Consequently, the medium is able to sustain the viability of oocytes and spermatozoa in culture, facilitate fertilisation of an oocyte with a spermatozoa, and support pre-embryo development.

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Examples

Example 1: Endogenous production of Meiosis Activating Sterol in mouse cumulus enclosed oocytes stimulated with FSH and EGF during culture

A total of 20 immature mice were stimulated with exogenous gonadotropins (7.5 IU per
5 mouse; Gonadoplex, Leo, Ballerup, Denmark). Forty-eight hours after injection with gonadotropins the mice were sacrificed and the ovaries removed. Oocytes were recovered by puncturing individual follicles and collected in a culture medium (α-MEM, Gibco, BRL) supplemented with 3 mg/ml bovine serum albumin, 5 mg/ml human serum albumin, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 4 mM
10 hypoxanthine. A total of around 650 cumulus enclosed oocytes were isolated. The cumulus enclosed oocytes (CEO) were randomly divided into two equal pools consisting of 325 CEO each. One of the pools served as a control and was cultured in a control medium without the addition of FSH and EGF. The other served as a test and the oocytes were cultured in the presence of 25 IU/L human FSH (Gonal F, Serono Nordic, Sweden)
15 and 2 ng/ml EGF (Sigma, , USA). In addition, the test and the control culture both received radioactive labeled mevalonate (^{3}H-mevalonat) (90 μCi ^{3}H-mevalonat; 38 Ci/mmol: NEN Life Science Products, Boston, MA, USA), which is a natural precursor for the production of sterols and steroids. The volume in each well was 400 μl.

20 After a culture period of 24 hours (37°C, 100% humidity in 5% CO_2 in air) steroids and sterols were removed from the media and the CEO, by an organic extraction after acidification with 50μ l 0.3 M Na_2 PO_4, pH 1.0. A mixture of n-heptane:isopropanol (3:1 v/v) in a five-fold surplus was added and shaked vigorously for 3 hours. The organic phase was isolated and lanosterol, FF-MAS, T-MAS, cholesterol and progesterone were
25 purified by two consecutive HPLC steps (for details see Baltzen and Byskov 1999) to involving a primary straight phase column (ChromSpher-TM Si, 5μm, 250×4.6mm; mobile phase: 99.5% n-heptane, 0.5% isopropanol (v/v); flow-rate: 1.0 ml/min; temp: 28°C) and a second reverse phase column (LiChrosper-TM RP-8,5μm, 250×4.6mm; mobile phase: 92.5% acetonitrile, 7.5% water (v/v); flow rate: 1.0 ml/ml; temp: 40°C). The fractions
30 selected for further purification on the second column were dried and reconstituted in 100μl acetonitrile. Fractions containing lanosterol, FF-MAS and T-MAS were prior to loading on the second HPLC column spiked with cold standards of the respective substances in order to avoid loss of radioactive sterols due to unspecific binding.

Radioactivity was monitored in each of the purified fractions. The biosynthesis of
lanosterol, FF-MAS, T-MAS and cholesterol were quantified by measuring the
incorporated radioactivity. The effect of FSH/EGF was evaluated by calculating the ratio of
the radioactivity per CEO in test and control cultures.

5

Table 1 Ratio of radioactivity incorporated in sterols and steroids between CEO cultured
with and without FSH and EGF stimulation

Lanosterol	FF-MAS	T-MAS	Cholesterol	Progesterone
0.9	4.0	2.2	0.2	2.1

These results presented in table 1 demonstrate that the presence of FSH and EGF in
10 cultures of CEO causes the accumulation of endogeneous produced FF-MAS and T-MAS
by attenuating the conversion of T-MAS to cholesterol. FSH and EGF causes a specific
effect on the accumulation of FF-MAS and T-MAS as illustrated by the fact that the ratio
for lanosterol is almost unaffected and the production of cholesterol is reduced around five
times. However, FSH and EGF also stimulate the production of progesterone.

15

Example 2: Significant implantation rate of human pre-embryos cultured in the presence of FSH and EGF

Fifty-seven women undergoing in-vitro fertilization (IVF) treatment were inrolled in this
study in order to evaluate the effect of enriching the oocyte culture medium with FSH and
20 EGF. The following inclusion criteria were used for this study: i) age of woman below 40
years, ii) regular menstruation with intervals between 26 and 32 days, iii) normal pre-
treatment levels of gonadotropins (less than 10 IU/L of FSH and LH) and iiii) more than 12
preovulatory follicles at the time of oocyte recovery. Exclusion criteria: i) cases in which
intracytoplasmic sperm injection (ICSI) was used, ii) infertility caused by endocrine
25 abnormality, e.g. polycystic ovaries. For pituitary down-regulation all women received
Gonadotropin-releasing-hormone agonist (GnRHa; 0.5 mg buserelin per day) starting on
cycle day 21 and lasting 10-14 days. After achievement of ovarian quiesence, stimulation
with exogenous gonadotropins was initiated using a dose of 150-225 IU per day of
recombinant FSH (Gonal F, Serono Nordic, Sweden) together with buserelin (0.2 mg per
30 day). Ovulation was induced (10000 IU hCG) when more than two follicles measured
more than 17 mm in diameter. Oocytes were recovered using ultrasound guided
transvaginal aspiration. Half of the retrieved oocytes (every second) were cultured in a
standard medium without addition of FSH and EGF, and the other half in medium

supplemented with 2ng recombinant EGF/ml and 25 IU/L of recombinant FSH (Gonal F) for the first 24 hours. The standard medium consisted of Earles Balanced Salt Solution, supplemented with sodium bicarbonate ($NaHCO_3$ 2.1 g/L), pyruvate (11 mg/L), Human Serum Albumin (10 mg/ml), and penicillin (100 IU/L). Before use, the medium is adjusted

5   to 280 mOsmol/kg and to pH 7.3-7.4 and sterile filtered. After 24 hours during which the *in vitro* fertilisation takes place, the medium was removed and replaced with a standard medium which were similar in both groups. The culture was continued for 48 hours (to a total of 72 hours), after which replacement of pre-embryos was performed. Thereby, each woman served as her own control. The morphological best looking pre-embryo over-all

10   decided the group from which the pre-embryos for transfer were selected. A maximum of two pre-embryos were replaced, representing the over-all best looking pre-embryo plus next best looking pre-embryo from that group. The criteria for selecting the morphological best looking pre-embryo was done according to Yding Andersen et al. (1991). Consequently, pre-embryos from the two groups were not mixed making it possible later

15   unequivocally to decide from which group a pregnancy resulted.

Out of the 57 women three had oocytes which did not cleave *in vitro* and three had - due to the possible development of hyper-stimulation syndrome - all developed pre-embryos cryopreserved.

20

Table 2 Patient characteristics and results of ovarian stimulation of women undergoing IVF treatment

	Control medium	FSH/EGF medium
No. of women	24	27
Age (years)	31.4 (24-39)	30.7 (26-37)
Pre-treatment FSH levels (IU/l)	5.5±0.3	6.5±0.4
Pre-treatment LH levels (IU/l)	5.7±0.6	4.9±0.3
Fertility diagnosis:		
Tubal factor	15 (63%)	18 (68%)
Endometriosis	6 (25%)	4 (15%)
Idiopathic	3 (12%)	5 (18%)
No. of stimulation days	9.9±0.2	9.4±0.2
Exogenous FSH (IU)	1800±90	1725±90
Oestradiol cycle day 2 (pmol/l)	115±15	112±12
Oestradiol cycle day 9 (pmol/l)	2980±450	3860±500

Table 3 Outcome of IVF treatment and implantation rate of the derived pre-embryos.

	Control medium	FSH/EGF medium
No. of women	24	27
No. of oocytes	448	456
No. of fertilized oocytes	327 (73%)	356 (78%) NS
No. of pre-embryos	315 (70%)	338 (74%) NS
No. oocytes with ≥3 proneuclei	23 (5.1%)	24 (5.3%)
No. of pre-embryo replacements	24	27
No. of pre-embryos replaced	45	52
No. of positive pregnancy tests	6 (25%)	15 (56%) p<0.03
No. of clinical pregnancies	6 (25%)	13 (48%) NS
Implantation rate	6 (13%)	18 (38%) p<0.025

None of the above monitored parameters, presented in tables 2 and 3 differ significantly
between the two groups except for the implantation rate and the number of positive

5   pregnancy tests, which is significantly higher in the group of oocytes/pre-embryos
exposed to a medium containing FSH and EGF compared to that of the control.

The culture medium from 40 oocytes in each of the two groups were collected and pooled
to make up one pool from the control medium and one pool from the medium enriched

10   with FSH and EGF. The medium was collected directly from the four-well dishes using a
pasteur-pipette with manual suction. The steroids and sterols in the two pools were
extracted as described in Example 1.

Table 3a Quantification of MAS in culture medium in which oocytes have grown with or
15   without enrichment of FSH and EGF

	Lanosterol (ng)	FF-MAS (ng)	T-MAS (ng)	FF+T-MAS (ng)	Cholesterol (µg)	Progesterone (µg)
Control medium	<7	<4	<7	<11	15	1.7
FSH/EGF medium	90	117	94	210	13	5.4

The results given in table 3a show that the presence of FSH and EGF causes an
accumulation of FF-MAS and T-MAS, together with their precursor lanosterol, contrasting
the control medium where these substances cannot be detected. The concentration of

20   cholesterol remain unchanged, whereas the progesterone content also seems to increase
in the medium enriched with FSH and EGF.

·Example 3: Effect of FSH and EGF on resumption of meiosis of mouse oocytes cultured in vitro

Immature female mice (B6D2-F1 strain C57B1/2J) were kept under controlled light and
5 temperature conditions with free access to food and water. Ovarian stimulation was
performed when the mice weighed 10-16 grams and consisted of an intra-peritoneal
injection of Gonadoplex (Leo, Copenhagen, Denmark) containing (7,5 U/mouse). The
animals were killed by cervical dislocation 44-48 h later. The media used for the culture of
oocytes consisted of α-Minimum Essential Medium (α-MEM), with Earles Balanced Salt
10 Solution (EBSS), 4 mM hypoxantine (HX), 3 mg/ml Bovine Serum Albumin, 0,23 mM
pyruvate, 2 mM glutamine, 100 IU/ml penicillin and 100 mg/ml streptomycin. The ovaries
were recovered and placed in HX-medium where an initial cleaning and removal of
connective tissue was performed. Oocytes were isolated from the ovaries by puncturing
individual follicles using 25 gauge needles. Isolation of oocytes were performed in HX-
15 medium to prevent resumption of meiosis until the tests were ready to be performed. The
oocytes were then washed 3 times in control medium before the start of each experiment.
Cumulus enclosed oocytes were cultured separately in 4-well dishes (Nuncleon, Roskilde,
Denmark), 0.4 ml medium in each well containing control medium or medium
supplemented with FSH and/or EGF addition in a 100% humidified atmosphere of 5%
20 CO_2 with 95% air at 37°C. The culture period was 22-24 hours. By the end of the culture
period, germinal vehicle breakdown (GVBD) was scored by examining the oocyte in an
inverted microscope. The percentage of oocytes with GVBD per total number of oocytes
(%GVBD) was calculated.

Table 4 Effect of FSH and EGF on resumption of meiosis in mouse oocytes cultured in
25 vitro

	No. of oocytes	No. of GVBD	% GVBD
Control medium	154	29	19[a, b, c]
FSH (10 IU/l)	162	73	45[b]
EGF (0.5 ng/ml)	68	21	31[c, d]
FSH (10 IU/l) + EGF (0.5 ng/ml)	63	33	52[a, d]

Values with similar letter differ significantly $P<0.05$

The results presented in table 4 demonstrate that FSH and EGF enhance oocyte
maturation of mouse oocytes in vitro and that a combination of FSH and EGF seem to
give an enhanced effect.

30

Example 4: Effect of different FSH isoforms on resumption of meiosis of mouse oocytes cultured in vitro

The experiments were performed similar to those described in example 3.

5   FSH isoforms were isolated from human pituitary extracts using glycoprotein extraction followed by chromatofocusing. Before being applied to induce resumption of meiosis in mouse oocytes, the FSH isoforms were thoroughly dialysed against the control medium.

Each FSH isoform fraction were tested in serial dilutions and the dilution at which 50 % of
10   the oocytes resumed meiosis was determined (equivalent dose of 50% GVBD ($ED_{50\%}$ GVBD), the $ED_{50\%}$ GVBD value, and determination was repeated 3 to 5 times for each FSH isoform fraction. In each 4-well dish, one well always served as a control and contained oocytes cultured in the control medium without FSH. Each well contained between 30 and 40 CEO.

15

The FSH concentration of the isoform fractions tested was monitored using two methods:
1) a radio-immuno-assay employing specific rabbit anti-FSH antibodies and ^{125}I-labelled human FSH as a tracer. Bound and free radioactivity was separated by anti-rabbit immunoglobulin coated to dextran particles. Inter and intra-assay variation of a sample
20   containing 25 IU/l were 7% and 5% respectively, 2) a Chinese hamster ovary cell (CHO-cell) line which stably expresses the recombinant human FSH receptor and which upon stimulation with FSH releases cAMP. The amount of cAMP generated by the FSH isoform fractions was related to a standard. The inter- and intra-assay variation was 15% and 18% respectively.

Table 5 Effect of FSH isoform fractions on resumption of meiosis in mouse oocytes

FSH isoform fraction	Total no. of oocytes	Mean no. of oocytes per well	$ED_{50\%}$ GVBD RIA (IU/l)	$ED_{50\%}$ GVBD CHO-cell assay (ng/l)
Less acidic (pI 6.43-5.69)	517	34	6.4 ± 0.32^{a}	142 ± 7^{c}
Mid acidic (pI 5.62-4.96)	1088	37	6.1 ± 0.67^{a}	146 ± 16^{c}
Acidic (pI 4.69-3.75)	608	34	12.2 ± 0.74^{b}	215 ± 13^{d}

Values are mean ±SD. The $ED_{50\%}$ GVBD value is the concentration of FSH were 50% of the oocytes have entered meiosis after the 24h culture period. Within each column values with a similar letter are not significantly different and values with different letters show the
5 following levels of significance: a, b: $p<0.0005$; c,d: $p<0.001$.

The results demonstrated in table 5 indicate that less and mid-acidic FSH isoforms with a pI above 5.0 are significantly more potent in inducing mouse CEO's to resume meiosis *in vitro* than acidic FSH isoforms. Almost the double concentration of acidic FSH isoforms
10 was required to induce 50 % of the oocytes to resume meiosis compared to that of less and mid-acidic isoforms.

The actual levels of FSH isoform needed to induce 50 percent of the oocytes to resume meiosis obtained in this study compare favourably to studies in which unfractionated FSH
15 was used, where the $ED_{50\%}$ GVBD value was around 8 IU/l.

Whereas the mid-cycle of FSH in circulation reaches an amplitude of around 10-15 IU/l, the intrafollicular level of FSH which most likely represent the concentration to which the CEO is exposed to in vivo has in preovulatory follicular fluid from women just before
20 ovulation been measured to 4-6 IU/l. These data compare favourably to the effective doses of especially the less and mid-acidic isoforms in the mouse system, in which the oocytes are exposed to FSH for 20 to 24 hours.

As described in example 2, unfractionated FSH in a concentration of 25 IU/l was used in
25 order to secure an optimal stimulation of the human oocytes, also those oocytes which in vivo received a sub-optimal maturation or those which were immature at the time of retrieval, which could be expected to be less responsive to the FSH stimulus.

Example 5: Induction of resumption of meiosis and endogenous production
of Meiosis Activating Sterol in mouse cumulus enclosed oocytes exposed to
an inhibitor of sterol Δ14-reductase (AY9944-A-7) during culture

Immature female C57Bl/2J B6D2 mice (11-15 g) were stimulated with exogenous
5 gonadotropins (7.5 IU per mouse; Gonadoplex, Leo, Ballerup, Denmark). Forty-eight
hours after injection with gonadotropins, the mice were sacrificed and the ovaries
removed. Cumulus enclosed oocytes (CEO) were recovered by puncturing individual
follicles and collected in a culture medium (α-MEM, Gibco, BRL) supplemented with 3
mg/ml bovine serum albumin, 5 mg/ml human serum albumin, 2 mM L-glutamine, 100
10 IU/ml penicillin, 100 μg/ml streptomycin and 4 mM hypoxanthine (control medium).

Induction of resumption of meiosis in mouse cumulus enclosed oocytes exposed
to an inhibitor of sterol Δ14-reductase (AY9944-A-7) during culture.

Oocytes from 10-15 mice were pooled and randomly divided into the different test groups.
15 The tests were performed using 4-well dishes (Nunclon, Roskilde, Denmark) each well
containing 20-50 oocytes in 400 μl culture medium. Each dish had one well serving as
control and the three other wells as tests.

All test media were prepared using HX-medium. AY9944-A-7 (AY) was kindly provided by
20 Wyeth-Ayerst, Princeton, NJ, USA. AY has no structural similarities to MAS. Stock
solutions of AY9944 in water were stored at -20°C, and was added directly to the HX-
medium immediately prior to oocyte culture. AY was used in concentrations from 0.2 to
25μM.

25 The results presented in figure 2 show that AY stimulates oocyte maturation of CEO in a
dose-dependent way. Concentrations of 5, 10 and 25 μM increase GVBD significantly
compared to the controls ($p < 0.05$, $p < 0.001$ and $p < 0.001$, respectively).

Endogenous production of Meiosis Activating Sterol in mouse cumulus enclosed oocytes exposed to an inhibitor of sterol Δ14-reductase (AY9944-A-7) during culture.

Cumulus enclosed oocytes (CEO) were isolated and randomly divided into two equal
5   pools consisting of 250 CEO each. One of the pools served as a control and was cultured in a control medium without the addition of AY. The other served as a test and the oocytes were cultured in the presence of 10 μM AY. In addition, the test and the control culture both received radioactive labeled mevalonate (^{3}H-mevalonat) (90 μCi ^{3}H-mevalonat; 38 Ci/mmol: NEN Life Science Products, Boston, MA, USA), which is a natural precursor for
10 the production of sterols and steroids. The volume in each well was 400μl.

After a culture period of 24 hours, the steroids and sterols were extracted as described in Example 1.

15 The biosynthesis of lanosterol, FF-MAS, T-MAS and cholesterol were quantified by measuring the incorporated radioactivity. The effect of AY was evaluated by calculating the ratio of the radioactivity per CEO in test and control cultures.

Table 6 Ratio of radioactivity incorporated in sterols and steroids obtained from CEO
20 cultured with and without exposure to AY during the culture

Lanosterol	FF-MAS	T-MAS	Cholesterol	Progesterone
2.3:1	11:1	1:5.2	1:44	2.8:1

These results demonstrate that the presence of AY in cultures of CEO causes the accumulation of endogenous produced FF-MAS and T-MAS by attenuating the conversion of T-MAS to cholesterol. AY causes a specific effect on accumulation of FF-
25 MAS as illustrated by the fact that Lanosterol is only accumulated to a small extend, whereas the production of T-MAS and cholesterol is reduced around 5 and 44 times, respectively.

Eksample 6: Resumption of oocyte meiosis in mouse cumulus enclosed oocytes exposed to a cholesterol biosynthesis inhibitor, Amphotericin B, during culture

Amphotericin B is a medical product used against fungal infections by inhibiting
5   cholesterol biosynthesis. It has no structural similarities to MAS. Its effect on resumption
of meiosis was tested on mouse cumulus enclosed oocytes, *in vitro*. Ovarian stimulation
was performed on immature female mice and the cumulus enclosed oocytes (CEO)
isolated as described in Example 3.

10   Cumulus enclosed oocytes were cultured separately in 4-well dishes (Nuncleon, Roskilde,
Denmark), 0.4 ml medium in each well containing control medium or medium
supplemented with Amphotericin B (Bristol-Myers Squibb) in a 100% humidified
atmosphere of 5% CO_2 with 95% air at 37°C.

15   The effect of Amphotericin in concentrations of 0.6 and 1.2 µg/ml was evaluated after 22
hours in culture.

In another series of experiments, i.e. priming of CEO, the culture period with
Amphotericin, priming period, varied from 5 min. to 240 min. in test medium with 1.2 µg/ml
20   Amphotericin. After the priming period, the CEO were transferred to control medium and
the culture continued for a total of 22 hours.

By the end of the culture period, germinal vehicle breakdown (GVBD) was scored by
examining the oocyte in an inverted microscope. The percentage of oocytes with GVBD
25   per total number of oocytes (%GVBD) was calculated.

Table 7 Effect of amfotericin on resumption of meiosis in mouse cumulus enclosed oocytes *in vitro*

Amfotericin (µg/ml)	No. of oocytes	No. of GVBD	%GVBD
Control	176	28	16
0.6	94	26	28
1.2	121	92	76

Table 8 Effect of amfotericin (1.2µg/ml) priming (i.e. the period in which the CEO's are exposed) on resumption of meiosis in mouse cumulus enclosed oocytes *in vitro*

Time (minutes)	No. of oocytes	No. of GVBD	%GVBD
Control	175	26	15
5	98	21	21
10	88	26	29
30	116	42	36
120	50	25	50
240	49	34	69

The results presented in tables 7 and 8 demonstrate that Amphotericin causes a dose
5   dependent resumption of oocyte meiosis.

Example 7: Method used for electing MAS and MAS analogues

Oocytes were obtained from immature female mice (C57BL/6J x DBA/2J F1, Bomholtgaard, Denmark) weighing 13-16 grams, that were kept under controlled
10   temperature (20-22 °C), light (lights on 06.00-18.00) and relative humidity (50-70%). The mice received an intra-peritoneal injection of 0.2 ml gonadotropins (Gonal-F, Serono) containing 20 IU FSH and 48 hours later the animals were killed by cervical dislocation. The ovaries were dissected out and the oocytes were isolated in Hx-medium (see below) under a stereomicroscope by manual rupture of the follicles using a pair of 27 gauge
15   needles. Spherical oocytes displaying an intact germinal vesicle (hereinafter designated GV) were divided in cumulus enclosed oocytes (hereinafter designated CEO) and naked oocytes (hereinafter designated NO) and placed in a-minimum essential medium (α-MEM without ribonucleosides, Gibco BRL, Cat. No. 22561) supplemented with 3 mg/ml bovine serum albumin (BSA, Sigma Cat. No. A-7030), 5 mg/ml human serum albumin (HSA,
20   Statens Seruminstitut, Denmark), 0.23mM pyruvate (Sigma, Cat. No S-8636), 2 mM glutamine (Flow Cat. No. 16-801), 100 IU/ml penicillin and 100 mg/ml streptomycin (Flow, Cat No. 16-700). This medium was supplemented with 3 mM hypoxanthine (Sigma Cat. No. H-9377) and designated Hx-medium. The oocytes were rinsed three times in Hx-medium and oocytes of uniform size were divided into groups of CEO and NO. CEO and
25   NO were cultured in 4-well multidishes (Nunclon, Denmark) in which each well contained 0.4 ml of Hx-medium and the compound to be tested in a concentration of 10 mM. One control well (i.e., 35-45 oocytes cultured in identical medium with no addition of test compound) was always cultured simultaneously with 3 test wells (35-45 oocytes per well

supplemented with test compound). The oocytes were cultured in a humidified atmosphere of 5% CO_2 in air for 24 hours at 37°C. By the end of the culture period, the number of oocytes with GV, GVB and polar bodies (hereinafter designated PB), respectively, were counted using a stereo microscope (Wildt, Leica MZ 12). The

5 percentage of GVB, defined as percentage of oocytes undergoing GVB per total number of oocytes in that well, was calculated as: % GVB = ((number of GVB + number of PB)/ total number of oocytes) X 100.

Claims

1. Use of a culture medium comprising
   - at least one meiosis activating sterol (MAS), a MAS being any sterol in the metabolic
     pathway between lanosterol and cholesterol,
5 - a MAS analogue, or
   - an additive or additives capable of endogenous stimulation of the accumulation of at
     least one MAS for *in vitro* fertilisation, the from the *in vitro* fertilisation resulting pre-embryo
     having an improved implantation rate *in vivo*.

10 2. Use of at least one MAS, a MAS analogue, or an additive or additives capable of
    endogenous stimulation of the accumulation of at least one MAS in cumulus enclosed
    oocytes for the preparation of a culture medium for *in vitro* fertilisation, the from the *in vitro*
    fertilisation resulting pre-embryo having an improved implantation rate *in vivo*.

15 3. Use according to claim 1 or 2, wherein MAS is selected from the group consisting of
    FF-MAS, T-MAS, 1-methyl-zymosterol, and zymosterol.

    4. Use according to any of the preceding clams, wherein MAS is FF-MAS.

20 5. Use according to any of the preceding clams, wherein MAS is T-MAS.

    6. Use according to claims 1 or 2, wherein the culture medium comprises a MAS
    analogue.

25 7. Use according to claims 1 or 2, wherein the additive or additives leads to a ratio of at
    least 2 between the relative content of MAS in cumulus enclosed oocytes cultured in the
    presence of the additive or additives, the relative content of MAS in cultured cumulus
    enclosed oocytes being determined by stimulation female mice with exogenous
    gonadotropins 48h prior to removal of the ovaries from the mice and recovering cumulus
30 enclosed oocytes from the ovaries by puncturing individual follicles and culturing the
    recovered cumulus enclosed oocytes in an α-MEM medium supplemented with 3mg/l
    bovine serum albumin, 5 mg/l human serum albumin, 2mM L-glutamin, 100IU/ml
    penicillin, 100μg/ml steptomycin, 4mM hypoxanthine and ^{3}H-mevalonat for 24h at 37°C,
    100% humidity and 5% CO_2 in air, followed acidification with 50μl 0.3M Na_2PO_4 pH=1,

organic extraction three times with a five-fold surplus of n-heptane:isopropanol (3:1 v/v),
purification of MAS from the organic phase by HPLC and determination of the ratio of
radioactivity per cumulus enclosed oocyte between cumulus enclosed oocytes cultured in
the presence of the additive or additives and cumulus enclosed oocytes cultured without
5   the presence of the additive or additives.

8. Use according to claim 7, wherein the additive is selected from the group consisting of
gonadotropins such as FSH and analogues, growth hormones such as EGF and
analogues, compounds inhibiting sterol Δ14-reductase such as AY9944-A-7, compounds
10 inhibiting 4-demethylase converting T-MAS to Zymosterol, compounds activating
cytochrome P450 lanosterol 14α -demethylase and compounds with an amphotericin like
effect.

9. Use according to claim 8, wherein the additive is a combination of a gonadotropin and a
15 growth hormone.

10. Use according to claim 9, wherein the additive is a combination of EGF and FSH.

11. Use according to claim 8, wherein the additive is EGF.
20
12. Use according to claim 8, wherein the additive is FSH.

13. Use according to claim 10 or 12, wherein FSH is an FSH isoform with an isoelectric
point above 5.0.
25
14. Use according to any of claims 10, 12 or 13 wherein the FSH is derived from naturally
occurring FSH such as FSH extracted from urine, or from recombinant FSH.

15. Use according to any of claims 10 or 12-14, wherein the concentration of FSH is
30 between 2 and 200IU FSH/l.

16. Use according to any of claims 10 or 11, wherein the concentration of EGF is between
1 and 10ng EGF/ml.

35 17. Use according to claims 7 or 8, wherein the additive is amphotericin.

18. Use according to any of the preceding clams, wherein the *in vitro* fertilisation is *in vitro* fertilisation of human oocytes.

5   19. A method for *in vitro* fertilisation comprising the step of: exposing and culturing one or more MII oocytes with spermatozoa in a culture medium, the culture medium comprising at least one MAS, a MAS analogue, or an additive or additives capable of endogenous stimulation of the accumulation of at least one MAS; the exposure and culturing lasting until zygotes and/or pre-embryos are formed.

10

20. A method for *in vitro* fertilisation comprising the steps of:

(a) culturing one or more GV oocytes in a culture medium, the culture medium comprising at least one MAS, a MAS analogue, or an additive or additives capable of endogenous stimulation of the accumulation of at least one MAS; hereby forming one or more MII

15   oocytes;

(b) exposing and culturing the one or more MII oocytes of step (a) with spermatozoa in a culture medium, the culture medium comprising at least one MAS, a MAS analogue, or an additive or additives capable of endogenous stimulation of the accumulation of at least one MAS; the exposure and culturing lasting until zygotes and/or pre-embryos

20   are formed.

21. A method according to claims 19 or 20, wherein the MII oocytes are cumulus enclosed oocytes.

25   22. A method according to any of claims 19-21, wherein the culture medium comprises an additive or additives capable of endogenous stimulation of the accumulation of at least one MAS.

23. A method according to any of claims 19-21, wherein the culture medium comprises

30   FF-MAS, T-MAS, 1-methyl-zymosterol, and/or zymosterol.

24. A method according to any of claims 19-21, wherein MAS is FF-MAS.

25. A method according to any of claims 19-21, wherein MAS is T-MAS.

35

26. A method according to any of claims 19-21, wherein the culture medium comprises a MAS analogue.

27. A method according to any of claims 19-26, wherein the oocytes are nude.

5

28. A method according to any of claims 19-27, wherein the culture medium comprises at least one MAS or at least one MAS analogue.

29. A method according to any of claims 19-18, wherein 1-15 oocytes are cultured and
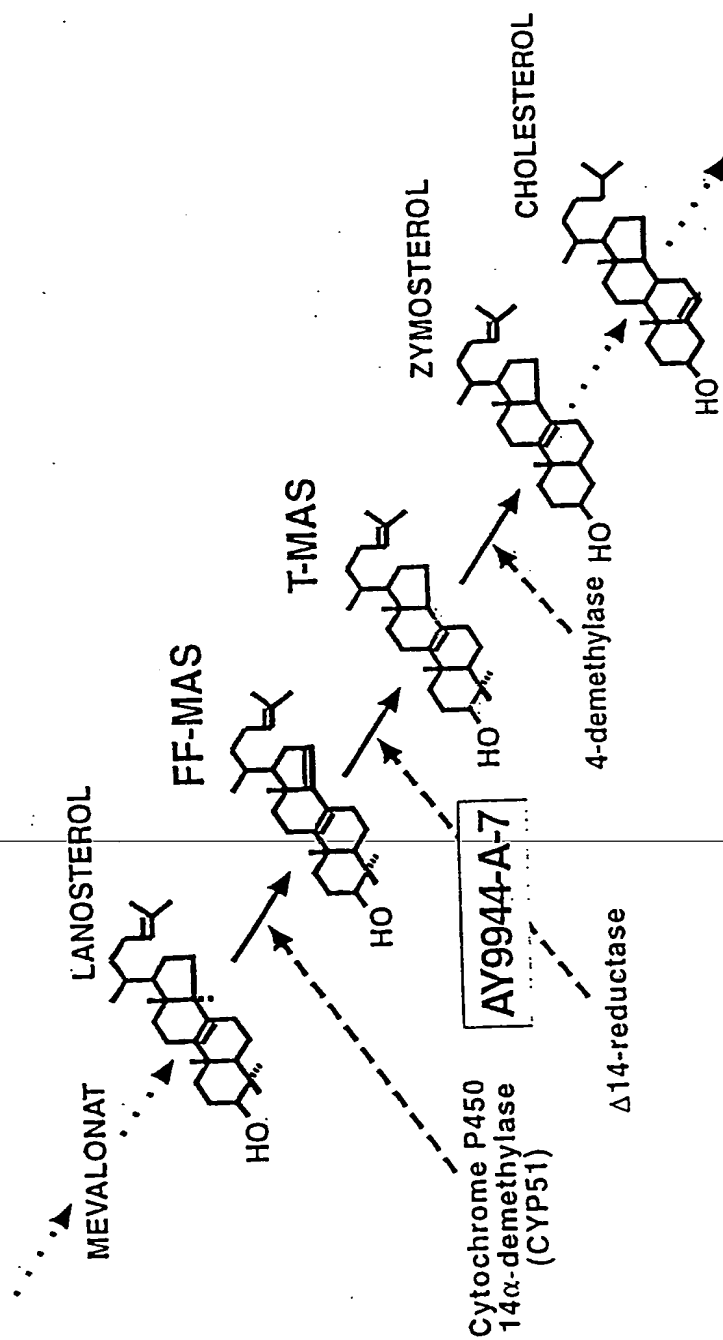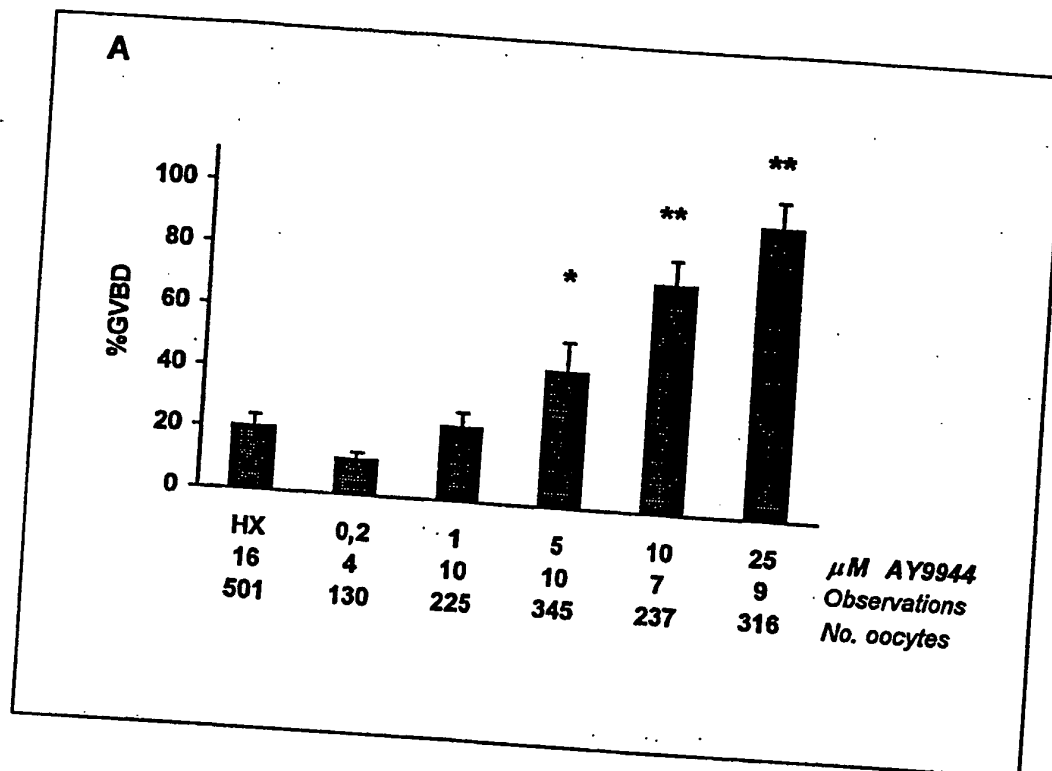10 exposed together.

Fig. 1

Fig. 2